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Kunji, ERS; Slotboom, DJ; Poolman, B

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## Review

# *Lactococcus lactis* as host for overproduction of functional membrane proteins

Edmund R.S. Kunji<sup>a,\*</sup>, Dirk-Jan Slotboom<sup>a</sup>, Bert Poolman<sup>b</sup><sup>a</sup>MRC Dunn Human Nutrition Unit, Hills Road, CB2 2XY Cambridge, UK<sup>b</sup>Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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## Abstract

*Lactococcus lactis* has many properties that are ideal for enhanced expression of membrane proteins. The organism is easy and inexpensive to culture, has a single membrane and relatively mild proteolytic activity. Methods for genetic manipulation are fully established and a tightly controlled promoter system is available, with which the level of expression can be varied with the inducer concentration.

Here we describe our experiences with lactococcal expression of the mechanosensitive channel, the human KDEL receptor and transporters belonging to the ABC transporter family, the major facilitator superfamily, the mitochondrial carrier family and the peptide transporter family. Previously published expression studies only deal with the overexpression of prokaryotic membrane proteins, but in this paper, experimental data are presented for the overproduction of mitochondrial and hydrogenosomal carriers and the human KDEL receptor. These eukaryotic membrane proteins were expressed in a functional form and at levels amenable to structural work.

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**Keywords:** *Lactococcus lactis*; Nisin A expression system; Eukaryotic and prokaryotic membrane protein; Transport; Binding assay

## 1. Introduction

In this paper, we describe the overexpression of integral membrane proteins in the Gram-positive lactic acid bacterium *Lactococcus lactis*. The lactococcal expression system is an excellent alternative to others for the overproduction of membrane proteins and their isolation from the cytoplasmic membrane. The properties that make this bacterium very

suitable for protein expression and purification are the following: (i) growth of lactococci is rapid, proceeds to high cell densities and does not require aeration; (ii) most strains are multiple amino acid auxotrophs, making them suitable for incorporation of labels; (iii) efficient transformation systems are available and constructed plasmids are stable; (iv) strong and tightly regulated promoter systems are available, which allow expression of toxic gene products; (v) the expression levels of membrane proteins are highly reproducible; (vi) the small genome size of the organism causes little redundancy, which facilitates complementation studies and allows for easier purification; (vii) expressed membrane proteins are targeted exclusively to the cytoplasmic membrane and, so far, inclusion bodies have not been observed; (viii) the organism has mild proteolytic activity; (ix) the organism has a single (cytoplasmic) membrane, which allows direct use of ionophores, ligands and metabolic inhibitors to study the activity of the protein in whole cells; and (x) membrane proteins are readily solubilized from the membrane in a wide range of mild detergents.

Different expression systems are available for regulated and constitutive expression in *L. lactis* [1], but the one most

**Abbreviations:** CBB, Coomassie brilliant blue; KDEL, Lys–Asp–Glu–Leu; HDEL, His–Asp–Glu–Leu; CDM, chemically defined medium; ER, endoplasmic reticulum; SDS-PAA, sodium dodecyl sulfate-polyacrylamide; AAC, ADP/ATP carrier; ABC, ATP binding cassette; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ISO, membrane vesicles with inside–out orientation; RSO, membrane vesicles with right-side–out orientation; TM, transmembrane; MFS, major facilitator superfamily; MS, mechanosensitive channel; PDR, pentanucleotide direct repeat; SBD, substrate-binding domains

\* Corresponding author. Tel.: +44-1223-252850; fax: +44-1223-252875.

E-mail address: [ek@mrc-dunn.cam.ac.uk](mailto:ek@mrc-dunn.cam.ac.uk) (E.R.S. Kunji).

<sup>1</sup> Part of this work was performed by ERSK in the MRC Laboratory of Molecular Biology, Hills Road, CB2 2QH, Cambridge, UK.

often used is based on the lactococcal nisin gene cluster, consisting of the *nisA* promoter and the two-component regulatory trans-acting factors NisR and NisK [2,3]. The system has also been made suitable for expression in other organisms, including *Enterococcus faecalis* and *Lactobacillus plantarum*. An equivalent system based on the subtilisin gene cluster of *Bacillus subtilis* has also been constructed (M. Kleerebezem, unpublished).

The use of *L. lactis* as host for membrane protein expression is not widely known, so we have given an extended introduction to describe how the system works and which plasmids are available for expression trials. In addition, we review the use of *L. lactis* for the overproduction of bacterial membrane proteins. Experimental data are then presented for the first use of *L. lactis* for the overproduction of eukaryotic membrane proteins.

### 1.1. Properties and growth of the organism

Lactococci are aerotolerant multiple amino acid auxotrophic lactic acid bacteria that are routinely cultured in complex broth (M17 or MRS) supplemented with the appropriate carbohydrate. At the optimal temperature, that is 29 °C, the organism grows with doubling times of 35–60 min, depending on the medium. During exponential growth, lactococci form chains of cells, like ‘beads on a string’, because separation occurs more slowly than division.

A chemically defined medium (CDM) is also available [4], which is particularly useful when one aims at the introduction of specific amino acid labels into proteins, for example the incorporation of <sup>13</sup>C isotopes for NMR studies. The commonly used MG1363 strain, and isogenic strains derived of MG1363, require Glu (or Gln), Leu, Ile, Val, His and Met, but for optimal growth the CDM needs to be supplemented further with Trp, Tyr, Phe, Arg, Lys and Pro. *L. lactis* strains are generally able to grow on a wide variety of carbohydrates, which are fermented to lactic acid leading to acidification of the medium. For small cultures without pH control, it can be useful to grow the organism on D-galactose plus L-arginine because co-metabolism leads to roughly equimolar acid and base production and an unaltered medium pH [5].

Small cultures from a few milliliters to liters are grown semi-anaerobically in M17 medium in tubes or bottles with 1% glucose and appropriate antibiotic. Overnight growth is sufficient for plasmid isolation, whereas cells cultured to late-exponential phase ( $A_{600} \sim 1$  without pH control) can be used for testing of expression levels and/or small-scale cell fractionation. For large-scale fermentation and subsequent isolation of membranes, the bacteria are preferentially cultured in fermentors with pH control at 6.8. In order to reach very high cell densities ( $A_{600} > 5$ ), the carbohydrate, amino acid and vitamin concentrations need to be doubled, otherwise essential nutrients may become limiting during growth [4]. In case of complex broth, one can double the concentration of medium components.

The concentrations of antibiotics needed for plasmid maintenance in *L. lactis* are 5 µg/ml for chloramphenicol and erythromycin. If the *L. lactis*/*E. coli* shuttle vectors are used in *E. coli*, 10 µg/ml of chloramphenicol is sufficient. Selection for erythromycin resistance in *E. coli* is generally not possible. *E. coli* BZ234 is relatively sensitive for erythromycin, but nonetheless high concentrations of the antibiotic are needed (200–500 µg/ml).

### 1.2. The nisin expression system

*L. lactis* possesses a biosynthetic pathway for the production of the biologically active peptide nisin A. This antimicrobial peptide inactivates a wide range of bacterial cells by permeabilizing their cytoplasmic membrane [6]. Nisin A is a so-called lantibiotic because the mature peptide contains thioether bridges called (β-methyl)-lanthionines. In addition to the lanthionine formation, the post-translational modifications of the ribosome-synthesized 57-residue peptide precursor of nisin A include the introduction of uncommon, unsaturated residues like dehydroalanine and dehydrobutyrine, and the cleavage of the pre-sequence, which results in a mature peptide of 34 residues [3]. The nisin A biosynthetic pathway is encoded by the *nisABT-CIPRKEFG* gene cluster [7]. Relevant for the development of the nisin expression system have been the *nisA* promoter and *nisRK* genes, encoding a two-component regulatory system. The cells are protected against nisin A by an immunity factor, encoded by *nisI*, and an ATP binding cassette (ABC) transporter encoded by the genes *nisFEG*.

The *nisA* promoter is composed of an – 10 element that is preceded by two pentanucleotide direct repeats (PDRs) rather than the conventional – 35/– 10 regions (Fig. 1). The PDRs are centered at – 26 and – 37 and required for transcriptional activation of the *nisA* promoter [2]. The PDRs function as the binding site for NisR, the response regulator that acts as transcription activator when phosphorylated. The other trans-acting factor of the *nisA* promoter is the sensor-kinase NisK, which autophosphorylates upon binding of nisin A to the N-terminal (largely extracellular) domain [7,8]. This two-component regulatory system, also termed NICE for nisin-controlled expression, has been transferred to a range of (lactic acid) bacteria [9–11], but the system has mainly been used for protein expression in the native host *L. lactis*. For the purpose of protein expression in *L. lactis*, the *nisRK* genes have been integrated into the *pepN* locus of the chromosome of a MG1363 derivative, called NZ9000. High-level expression is obtained by adding nisin A to a culture of *L. lactis* NZ9000, carrying the gene of interest downstream of the *nisA* promoter on a plasmid. The concentrations of nisin A needed for induction are relatively low and growth of the organism is not inhibited even when the NisI plus NisFEG immunity system is absent. Instead of using purified nisin A, one can also use the culture supernatant of the nisin-producing strain NZ9700 [7]. The culture supernatant is most often used at a titer of 1 in 1000 and can be stored at

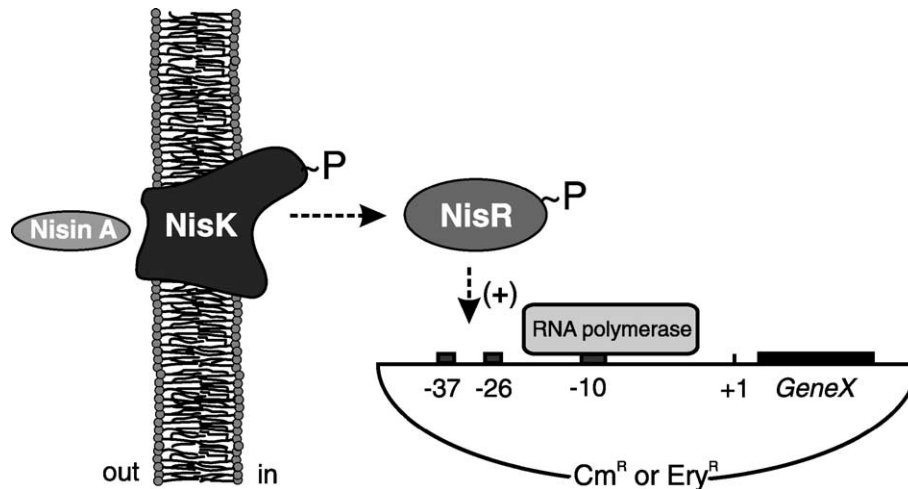


Fig. 1. Schematic representation of the nisin A expression system. The genes encoding NisK and NisR are integrated in the chromosome of *L. lactis* NZ9000. A plasmid vector with the gene of interest downstream of the  $-37/-26/-10$  *nisA* promoter region is electroporated into *L. lactis* NZ9000 and plasmid selection is done with either chloramphenicol ( $\text{Cm}^R$ ) or erythromycin ( $\text{Ery}^R$ ). Purified nisin A or the culture supernatant from a nisin A-producing strain is used to induce the expression of the gene of interest.

$-20^\circ\text{C}$  for prolonged periods of time. The optimal inducing activity has to be found empirically by testing different nisin A concentrations (0.01–1 ng/ml) or culture supernatant dilutions (from 100 to 10,000-fold), and by varying the induction times from 15 min up to several hours. An example of nisin A-induced heterologous expression of the xyloside transporter (XylP) from *Lactobacillus pentosus* is shown in Fig. 2.

The plasmid vectors most often used for expression of genes in *L. lactis* are based on the pSH71 replicon carrying the chloramphenicol resistance gene, which can replicate in a range of Gram-positive bacteria as well as *E. coli* [2,9]. The vector most frequently used is pNZ8048, which has a unique *NcoI* site for translational fusions; the site is followed by

*PstI*, *SphI*, *BanI*, *KpnI*, *SpeI*, *XbaI*, *SstI* and *HindIII* sites for directional cloning. Many derivatives of this vector have been constructed for in-frame fusion of the structural gene to nucleotide sequences that encode poly-histidine tags with or without specific protease sites [12,13]. Various transcriptional fusion vectors are also available, but these give lower expression levels [2]. The transcriptional fusion vectors, however, seem to be even more tightly regulated, which may be advantageous when genes coding for potentially toxic products are expressed. It is our experience that ligation mixtures of the pGK13- or pNZ8048-derived vectors and the gene of interest can best be transformed to *L. lactis* strains MG1363 or NZ9000 directly. Generally, a higher number of transformants with the correct insert is observed than with *E. coli*. The *nisA* promoter system is very tight in *L. lactis* and little or no expression is observed in the absence of inducer (Fig. 2B).

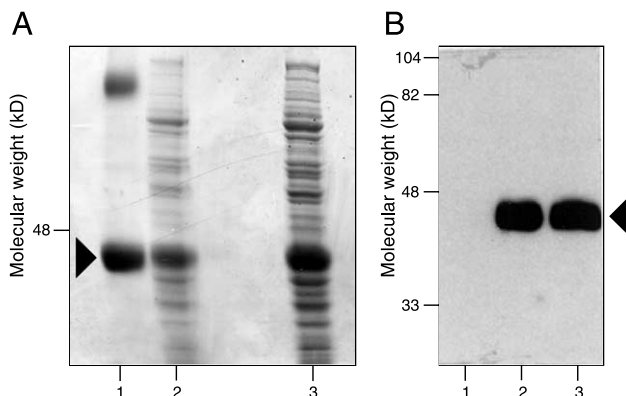


Fig. 2. A CBB-stained SDS-PAA gel and immunoblot of membranes from *L. lactis* NZ9000/p8048XylP overexpressing the XylP protein from *L. pentosus*. The cells were induced with 1 ng/ml nisin and membrane vesicles with inside-out orientation were isolated as described [17]. (A) CBB-stained SDS-PAA gel of Ni-NTA purified XylP protein (lane 1; mono- and dimeric protein is visible); membrane vesicles from cells overexpressing the *xylP* gene (lanes 2 and 3). (B) Western blot of membrane proteins from cells that were induced for 0 (lane 1), 1 (lane 2) or 2 hours (lane 3).

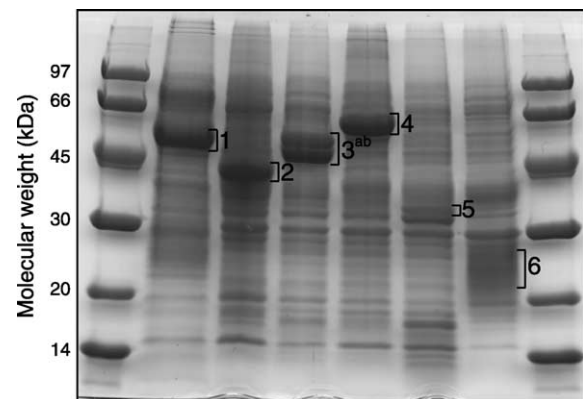


Fig. 3. A CBB-stained SDS-PAA gel of membrane proteins of strain NZ9000 overproducing XylP (2), the OpuA proteins (A and BC; 3<sup>ab</sup>), LmrA (4), LmrP (5) or MscL (6). LacS overexpressed in *S. thermophilus* is shown for comparison (1). For details of the proteins, see Table 1.

### 1.3. Expression of prokaryotic membrane proteins

Many prokaryotic membrane proteins have been successfully expressed in *L. lactis*, including ABC transporters for solute uptake or drug excretion, major facilitator superfamily (MFS) transporters for solute uptake and drug excretion, mechanosensitive channels, lipoproteins, and peptide transporters of the PTR family (Table 1 and Fig. 3). The LmrA drug efflux pump is a so-called ABC half-transporter consisting of an N-terminal transmembrane domain fused to an ABC domain. Two of these proteins form the functional (dimeric) complex. By contrast, the ABC units of the OpuA and GlnP<sub>2</sub>Q<sub>2</sub> transporters are separate gene products, but here one or two externally located substrate-binding domains (SBDs) are fused to the transmembrane domain. In the case of OpuA, a single SBD is fused to the C-terminus of the transmembrane domain [13], whereas in case of GlnP<sub>2</sub>Q<sub>2</sub>, two such domains are fused in tandem to the N-terminus of the

GlnP protein. The two SBDs of the GlnP protein are preceded by a typical signal sequence, which is cleaved off following insertion of the protein complex in the membrane (G. Schuurman-Wolters and B. Poolman, unpublished). Despite the very different domain organisations of these three ABC transporters (LmrA, OpuA and GlnP<sub>2</sub>Q<sub>2</sub>), each could be amplified in *L. lactis* in a fully functional state. There is less variation in the domain organisation of the ion-linked transporters that we have studied, but in one case, the LacS protein from *Streptococcus thermophilus*, a large cytoplasmic regulatory domain, known as IIA, is present at the C-terminus [14]. Also in this case no indications were obtained that the extramembraneous domain is either beneficial or inhibitory for the expression of the protein in *L. lactis*. All membrane proteins listed in Table 1 have been isolated from *L. lactis*, purified to homogeneity, and reconstituted in the fully functional state in liposomes with exception of the ADP/ATP transport proteins from *Rickettsia prowazekii* and the

Table 1  
Amplified expression of membrane proteins using the nisin promoter

System	Type of system <sup>a</sup>	Number of amino acids <sup>b</sup>	Number of TM $\alpha$ -helices	Extramembraneous domains <sup>c</sup>	Quaternary structure <sup>d</sup>	Organism <sup>e</sup>	Expression level <sup>f</sup>
<i>Prokaryotic membrane proteins</i>							
LmrA	ABC efflux pump	590	6	ABC domain at C-terminus (inside)	homodimer	<i>L. lactis</i>	~ 30%
XylP	MFS xyloside transporter	479	12	no	homodimer	<i>Lb. pentosus</i>	~ 20%
OpuA	ABC transporter (two components)	573 (OpuABC) 407 (OpuAA)	8 (in OpuABC protein)	SBD at C-terminus (outside)	dimer (2 $\times$ 2 subunits)	<i>L. lactis</i>	>10% each component
DtpT	peptide transporter	463	12	no	unknown	<i>L. lactis</i>	~ 10%
MscL	MS channel	122	2	no	pentamer	<i>L. lactis</i>	5–10%
TlcA,B,C	ATP/ADP translocator	498	12	no	unknown	<i>R. prowazekii</i>	5–10%
LmrP	MFS efflux pump	408	12	no	monomer	<i>L. lactis</i>	~ 5%
GlnP <sub>2</sub> Q <sub>2</sub>	ABC transporter (two components)	714 (GlnP) 247 (GlnQ)	5 (in GlnP) <sup>g</sup>	two SBDs at N-terminus (outside)	dimer (2 $\times$ 2 subunits)	<i>L. lactis</i>	2–5% each component
LacS	MFS transporter	634	12	IIA domain at C-terminus (inside)	homodimer	<i>S. thermophilus</i>	1–2%
MleP	MFS transporter	425	11	no	unknown	<i>L. lactis</i>	1–2%
CitP	MFS transporter	442	11	no	unknown	<i>L. lactis</i>	1–2%
OppBCDF	ABC transporter (four components)	319 294	6 in OppB 6 in OppC	no	heterooligomer (4 components)	<i>L. lactis</i>	< 1%
<i>Eukaryotic membrane proteins</i>							
Erd2	KDEL-receptor	212	7	no	monomer	<i>H. sapiens</i>	< 0.1%
CTP1	mitochondrial carrier	299	6	no	homodimer	<i>S. cerevisiae</i>	~ 5%
AAC3	mitochondrial carrier	307	6	no	homodimer	<i>S. cerevisiae</i>	~ 5%
AAC <sub>hyd</sub>	hydrogenosomal carrier	308	6	no	homodimer	<i>N. patriciarum</i>	< 1%

<sup>a</sup> ABC, ATP-binding cassette; MFS, major facilitator superfamily; MS, mechanosensitive.

<sup>b</sup> Number of amino acids corresponds to the wild-type protein; the recombinant proteins used in the expression studies generally contain an N-terminal 10-histag plus enterokinase cleavage site or a C-terminal 6-histag plus factor Xa cleavage site.

<sup>c</sup> Extramembraneous domains refer to distinct hydrophilic domains such as the substrate-binding units and ATP-binding cassettes of ABC transporters; the substrate-binding domains are fused either to the N- or C-terminus of the transmembrane domain. The location of the extramembraneous domains is indicated between brackets [37].

<sup>d</sup> The quaternary structure has not been studied for every protein and for some, e.g., MscL from *L. lactis*, it is based on knowledge of homologous systems (for an overview, see Ref. [35], and for experimental evidence, see Ref. [36]).

<sup>e</sup> *L. lactis*, *Lactococcus lactis*; *Lb. pentosus*, *Lactobacillus pentosus*; *R. prowazekii*, *Rickettsia prowazekii*; *S. thermophilus*, *Streptococcus thermophilus*; *H. sapiens*, *Homo sapiens*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *N. patriciarum*, *Neocallimastix patriciarum*.

<sup>f</sup> Percentage of total membrane protein; the percentages represent an underestimate as the membranes are highly contaminated with soluble and peripheral membrane proteins (up to 50% of the protein). References are indicated in the text.

<sup>g</sup> GlnP is synthesized as a pre-protein and the first putative transmembrane segment is cleaved off (signal sequence), leaving a total of five transmembrane  $\alpha$ -helical segments.



oligopeptide transport system (OppBCDF). The difficulty of reconstituting a functional Opp complex probably relates to the complexity of the system. In addition to the assemblage of four components (OppB, C, D and F), a fifth component (substrate-binding lipoprotein) is required for function. The prokaryotic membrane proteins expressed in *L. lactis* have a varying number of transmembrane  $\alpha$ -helices (from 2 to 12), differ in their quaternary structure and can have the N- and/or C-terminus located on the inside or outside of the cell (see Table 1 for details).

The expression levels differ significantly for the various proteins, but were sufficient in each case to purify milligram amounts for biochemical and biophysical studies [12,13,15,16,36]. The protein levels as percentages of total membrane protein obtained with the *nisA* promoter system are summarized in Table 1. The values represent an underestimate, as significant amounts of cytosolic proteins remain associated with the membranes, in particular when membrane vesicles with right-side-out orientation are isolated. These 'loosely membrane-associated' proteins can be removed by treating the membranes with a combination of urea and/or cholate [17].

Although the expression of not every protein was studied in detail, some generalizations can be made. In most cases, the cells continued to grow after induction with Nisin A in late exponential growth phase. However, when the multi-drug transporters LmrA and LmrP were overexpressed, growth ceased almost instantaneously [12,15], while in the case of the mechanosensitive (MS) channel MscL the growth was reduced (Folgering and Poolman, unpublished). These differences are not surprising because overproduction of transporters for carbohydrates or compatible solutes is expected to be less detrimental than when the membrane is filled with broad specificity drug efflux systems or MS channels. Maximum expression levels were reached within 1 h, when the particular protein was overproduced to more than 5% of total membrane protein. In cases where the expression was lower, the levels could be improved by inducing for longer periods of time (2–4 h). Most of the expression studies have been performed with endogenous membrane proteins, but similar levels of expression can be reached with heterologous proteins, provided that the codon usage is compatible with AT-rich codon bias, like in case of the transporters from the prokaryotes *Lactobacillus* and *Rickettsia*.

#### 1.4. Expression of eukaryotic membrane proteins

To our knowledge, no cases have been reported in literature that use *L. lactis* as host for overexpression of eukaryotic membrane proteins. We have used the nisin expression system for the functional expression of the human KDEL receptor and transporters belonging to the mitochondrial carrier family. In the following sections, we discuss the methods that were used to produce and characterise these proteins. The results show that the lactococcal

expression system can be successfully used for the overproduction of eukaryotic membrane proteins.

## 2. Materials and methods

### 2.1. Construction of the expression vectors

The *aac* gene of *Neocallimastix patriciarum*, the *ctp1* and *aac3* genes of yeast and the human *erd2* gene were amplified by polymerase chain reaction (PCR) with *pfu-Turbo* DNA polymerase (Stratagene) to introduce a *NcoI*-compatible site at the start of the gene and an *XbaI* site after the stop codon. The DNA fragments were restricted and ligated into the pNZ8048 vector [2], previously restricted with *NcoI* and *XbaI*. The ligation mixtures were electroporated into electrocompetent *L. lactis* strain NZ9000 [17]. Vectors were isolated by miniprep (Qiagen), according to the instructions of the manufacturer with one alteration; 10 mg/ml lysozyme was added to the lysis buffer and the resuspended cells were incubated at 55 °C for 10 min prior to lysis. The constructs were confirmed by PCR, restriction analysis and sequencing (Cambridge Bioscience). The restriction enzymes and ligase were purchased from New England Biolabs.

### 2.2. Preparation of cells and membrane vesicles for transport assays

*L. lactis* cells were grown at 29 °C in M17 medium, supplemented with 1% glucose and 5  $\mu$ g/ml of chloramphenicol, till the  $A_{600}$  reached 0.8, after which the inducer was added to a final concentration of 1 ng/ml unless stated otherwise. For transport assays, cells were washed once with ice-cold phosphate-buffered saline (PBS) and resuspended in the same buffer at an  $A_{600}$  of 200.

Membrane vesicles of PBS-washed cells were prepared by mechanical disruption with a 2.2 kW Z+ cell disrupter (Constant Systems) at 30,000 psi. Whole cells were removed by centrifugation at  $8000 \times g$  for 10 min at 4 °C (Sorvall) and membranes were collected by centrifugation at  $140,000 \times g$  at 4 °C for 1 h. The isolated vesicles were washed in PBS, pelleted by ultracentrifugation (see above) and resuspended to a final concentration of 25 mg/ml.

Liposomes were prepared by mixing *E. coli* total lipid extract and egg yolk phosphatidylcholine in a 3:1 ratio (w/w) in 50 mM potassium phosphate (pH 7) containing 100 mM potassium chloride (buffer A) at a final lipid concentration of 20 mg/ml. To prepare membrane vesicles fused to liposomes and loaded with citrate, membranes (1 mg protein) were mixed with liposomes in a ratio of 1:5 protein to lipid (w/w) in 3 ml buffer A supplemented with 5 mM potassium citrate pH 7. The mixture was frozen in liquid nitrogen, slowly thawed at room temperature, sonicated in an ultrasonicator (Misonix) with a 3.2 mm tip at 15% output (20 cycles of 3 s with 10 s pauses) and stored in liquid

nitrogen. The mixture of fused membranes was slowly thawed at room temperature, extruded through 400 nm polycarbonate filters and pelleted by centrifugation in a Beckmann MLA130 rotor for 20 min at 55000 rpm and 4 °C. The pellet was resuspended in 200 µl buffer A containing 5 mM potassium citrate, 1 µM valinomycin and 1 µM nigericin. To remove external citrate, the suspension was applied to a 3.5 ml bed volume Sephadex G-75 gel filtration column, previously equilibrated with buffer A containing 1 µM valinomycin and 1 µM nigericin at 4 °C. The fused membranes were applied to the column, eluted in 800 µl of buffer A, and used immediately for transport assays.

### 2.3. Transport assays

Transport in whole cells was initiated by diluting 20 µl cells in 280 µl PBS containing 1.65 µM [8-<sup>14</sup>C]-adenosine 5'-triphosphate (Amersham Pharmacia Biotech). The experiments were performed at 25 °C with constant stirring. Transport in fused membrane vesicles was initiated by diluting 80 µl of the membrane vesicles (100 µg protein) in 300 µl buffer A containing 1 µM valinomycin and 1 µM nigericin and 1.52 µM [1,5-<sup>14</sup>C]-citric acid (Amersham Pharmacia Biotech). The experiments were performed at 30 °C with constant stirring. The uptake was quenched by adding 4 ml of ice-cold PBS, followed by immediate filtration over cellulose nitrate filters (0.45 and 0.2 µm pore size for cells and membranes, respectively). The filters were washed once with 2 ml of ice-cold PBS, transferred to a scintillation vial, and 2 ml of Ultima Gold AB scintillation liquid was added for label counting in a Packard TriCarb 2100 TR-liquid scintillation analyser.

### 2.4. Binding assays

Membrane vesicles with predominantly right-side-out and inside-out orientation were isolated according to established procedures [17]. Membranes (30 µg) were incubated in 100 µl 50 mM sodium acetate buffer, pH 5.0, in the presence of 137 nM of [<sup>3</sup>H]-YTSEHDEL (Cambridge Research Biochemicals) and 290 nM [<sup>14</sup>C]-sucrose (Amersham Pharmacia Biotech) at room temperature. After 15 min, the membranes were separated from the assay buffer by centrifugation for 10 min at 16,000 × g and 4 °C in a microfuge and the supernatants were immediately and carefully removed. The pellet was resuspended in 100 µl buffer and 1 ml of Ultima Gold AB scintillation liquid was added. A Packard TriCarb 2100 TR-liquid scintillation analyser was used to determine the levels of [<sup>3</sup>H] and [<sup>14</sup>C]-labels. Non-specific binding was determined by measuring the total amount of [<sup>14</sup>C]-sucrose in the pellet or by addition of 5000-fold excess of unlabelled YTSEHDEL. The pH dependency of binding was measured in 100 mM citrate-200 mM disodium *ortho*-phosphate buffers mixed to pH 4.0, 5.0, 6.0, 7.0 and 8.0. To determine the total number of accessible binding sites, 0.5% CHAPS was added as described before

[18]. This concentration was experimentally determined to be optimal for the used concentration of lactococcal membranes.

### 2.5. Protein electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide (SDS-PAA) gel electrophoresis was performed as described [19] and Western blotting was carried out using a semi-dry electroblotter (Hoefer) according to previously described methods [20]. The primary antiserum against *Neurospora crassa* AAC (kindly provided by Prof. Palmieri) or peptide 192–212 of the KDEL receptor (Calbiochem) was added at a titer of 1:20,000 and the anti-rabbit secondary antibodies or the anti-mouse secondary antibodies coupled to horse radish peroxidase (Sigma) at a dilution of 1:20,000. An ECL-kit (Pharmacia) was used for the detection of secondary antibodies coupled to horseradish peroxidase. Protein concentrations were determined with the BCA Protein Assay kit (Pierce).

## 3. Results

### 3.1. KDEL receptor

The KDEL receptor is a seven-helix integral membrane protein involved in the retrieval of proteins of endoplasmic reticulum (ER) from later stages of the secretory pathway. The lumen of the ER contains chaperones, isomerases and other enzymes, which aid the folding, assembly and maturation of secreted proteins. These ER residents carry a targeting signal in the form of a conserved C-terminal tetrapeptide sequence, typically Lys–Asp–Glu–Leu (KDEL) or His–Asp–Glu–Leu (HDEL). The sequence is recognized by the KDEL receptor in the Golgi apparatus, after which the receptor–ligand complex enters a retrograde transport pathway that returns them to the ER, where the proteins are released [21].

Expression vectors were constructed based on the *nisA* promoter system to overproduce the human KDEL receptor in *L. lactis*. Strains harbouring the expression and control vectors grew at the same rate ( $t_d \sim 50$  min), but after induction with nisin A, growth of the expression strain ceased within an hour after addition of the inducer (Fig. 4A). The observed increase in optical density after 1 h of induction was due to separation of cells from the chains (see above) and not to further division as could be inferred from cell counts. Thus, expression of the human KDEL receptor appeared to be toxic to lactococcal cells. This was confirmed when expression strains were plated onto nisin A and chloramphenicol-containing M17 plates. Colony counts established that only 1 in 10<sup>9</sup> colony-forming units survived induction.

Membrane vesicles isolated from the expression strain contained KDEL receptors, as was shown by Western blot analysis with antibodies raised against the C-terminal end

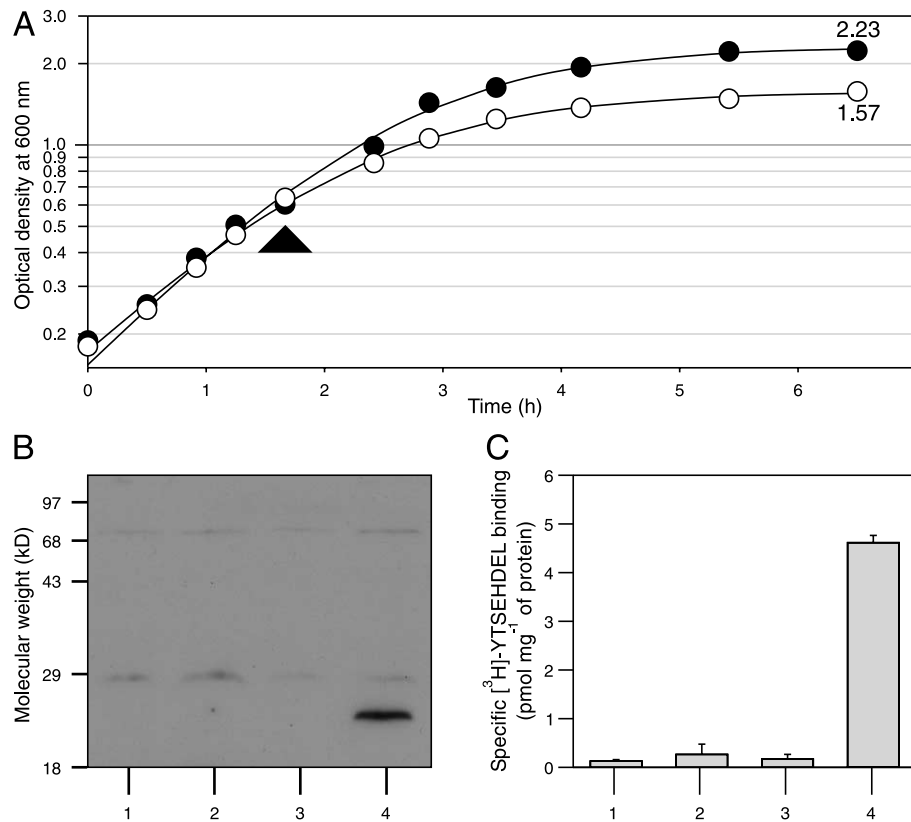


Fig. 4. Functional overexpression of the human KDEL receptor. (A) Growth curve of control strain NZ9000/pNZ and strain NZ9000/pNZ-*erd2* producing the KDEL receptor. The triangle indicates the time at which the inducer nisin A was added and the numbers indicate the final  $A_{600}$  measured for each strain. (B) Western blot probed with antibodies raised against peptide 192–212 of the KDEL receptor. (C) Binding studies carried out with isolated membranes of the uninduced (lane 1) and induced (lane 2) control strain NZ9000/pNZ and the uninduced (lane 3) and induced (lane 4) strain NZ9000/pNZ-*erd2*. Membranes were isolated by mechanical disruption and differential centrifugation. Approximately 30  $\mu$ g of total membrane protein was loaded in each lane. The binding studies were performed as described in the Materials and methods and carried out in duplicate.

of the receptor (molecular weight of  $\sim 23$  kDa) (Fig. 4B). No clear band was visible in SDS-PAA gels stained with Coomassie brilliant blue (CBB) or silver, indicating that the expression levels were likely to be low. Equilibrium spin assays with an [ $^3$ H]-YTSEHDEL peptide showed that the ligand only bound to membranes isolated from the induced expression strain, while no binding was observed in controls (Fig. 4C). It has been well-established that the binding of the ligand to rat Golgi membranes is pH-dependent [22] and this property was completely preserved when the KDEL receptor was expressed in lactococcal membranes, where it only bound the ligand at low pH values (Fig. 5A).

The ligand binds to the side of the receptor that is exposed to the lumen of the ER and Golgi. We have used this property to study the orientation of the KDEL receptor expressed in the lactococcal membranes. Membrane vesicles with predominantly right-side-out or inside-out orientation were isolated from the KDEL-expressing strain following established procedures that yield membranes with approximately 80% of the desired orientation [17]. To establish the total number of binding sites in each membrane preparation, CHAPS was added to permeabilise the vesicles without

solubilisation of the receptor [18]. In the case of the inside-out vesicles, the ligand binding increased by 3-fold when CHAPS was added (Fig. 5B). The specific binding in untreated right-side-out vesicles was much higher than that of inside-out vesicles and the increase was only 24% in the presence of CHAPS. Considering the fact that the membranes are never more than 80% of the desired orientation, these results show that the KDEL receptor is expressed almost exclusively with the binding site to the outside of the cell. These results are in agreement with the predicted orientation of the receptor based on the positive inside rule of Von Heijne [23].

Kinetic analyses showed that the expressed receptor had a dissociation constant for the ligand of  $82 \pm 31$  nM (data not shown), which corresponds well with the reported value of 78 nM for rat Golgi membranes [22]. The total yield of functional receptor was estimated to be around 6  $\mu$ g per liter of culture, on the basis of single point binding assays and estimates of membrane vesicle yields. This number corresponded to approximately 150 functional receptors per cell. These numbers are likely to be underestimates because of losses in the isolation of membrane vesicles, inaccessibility of binding sites, the relative high



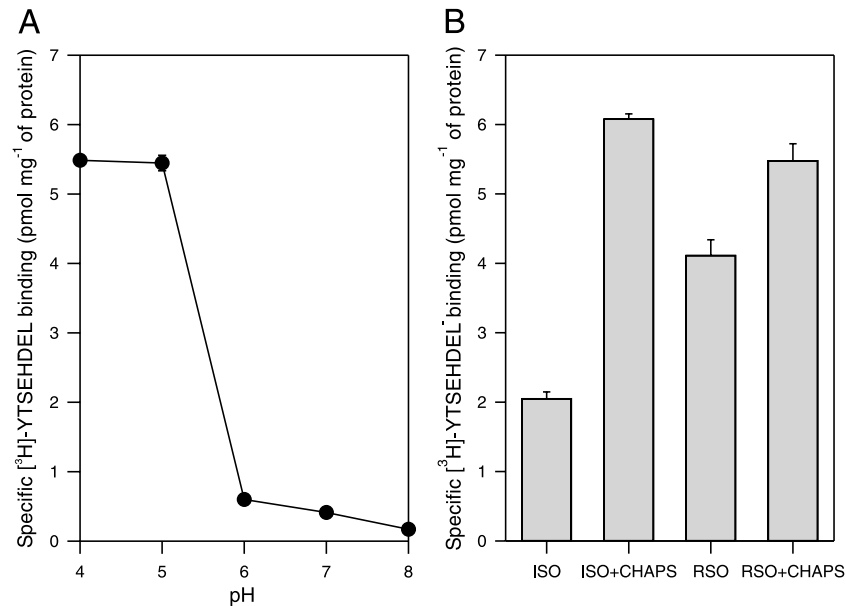


Fig. 5. pH dependency of ligand binding and orientation of the human KDEL receptor in lactococcal membranes. (A) Binding activity of the KDEL receptor for [<sup>3</sup>H]-YTSEHDEL was determined in 100 mM citrate/200 mM di-sodium *ortho*-phosphate buffer at different pH values using membrane vesicles with a predominantly right-side out orientation isolated from KDEL receptor-producing strain NZ9000/pNZ-*erd2*. The binding experiments were carried out in duplicate. (B) The orientation of the human KDEL receptor was determined using membrane vesicles with predominantly inside-out (ISO) or right-side-out (RSO) orientation from the KDEL receptor-producing strain NZ9000/pNZ-*erd2*. To probe inaccessible binding sites, 0.5% CHAPS was added to partially permeabilize the membranes [18].

off-rates and possible degradation of the peptide ligand by peptidases.

The KDEL receptor expression levels are low compared to all other membrane proteins tested in this system, which is most likely caused by the rapid death of the cells after induction. The expression levels, however, still compare favourably with levels of G-protein coupled receptors expressed in *E. coli* [24]. Moreover, it is much easier to scale-up fermentation of *L. lactis*, because the bacteria do not require aeration. We can culture *Lactococcus* in five 10-l bottles in a single Innova 4330 incubator shaker (New Brunswick Scientific). Recently published results show that the KDEL receptor can also be overexpressed in the cytoplasmic membrane of *E. coli* as a N-terminal fusion to green fluorescent protein (GFP), but the binding activity was not assayed [25].

### 3.2. Mitochondrial carriers

Mitochondrial carriers are integral membrane proteins that catalyse the transport of metabolites across the inner membrane of the mitochondria. These compounds are required for metabolic energy-generating pathways leading to oxidative phosphorylation, such as the conversion of pyruvate to acetyl CoA, the citric acid cycle, the malate shuttle and fatty acid oxidation. Members of the mitochondrial carrier family have tripartite repeats and signature motifs, and have also been found in organelles other than mitochondria [26–29]. We have recently identified an ADP/ATP carrier (AAC) in the primitive anaerobic fungus *N.*

*patriciarum*, which lacks mitochondria, but contains hydro-genosomes that carry out anaerobic energy metabolism leading to the production of hydrogen [28]. The protein was expressed in yeast mitochondria and uptake studies in membranes of isolated mitochondria confirmed the substrate and inhibitor specificity.

Prior to the work in yeast, the carrier was functionally expressed in *L. lactis* and characterised (Fig. 6). Western blots clearly showed that the carrier was expressed in lactococcal membranes to levels similar to those observed for expression of the carrier in yeast mitochondria (Fig. 6A). Transport experiments in whole cells showed that the uptake of ATP occurred only when the carrier was present in membranes (Fig. 6A and B). The substrate and inhibitor specificity of the carrier was established more quickly and easily in *L. lactis* with whole cell transport assays than with yeast mitochondria that required fusion of mitochondrial membranes with liposomes and the inclusion of 5 mM ATP to drive transport. [28]. When carboxy-atractyloside was added to the assay buffer, transport of ATP in whole cells was completely abolished (Fig. 6B). Carboxy-atractyloside specifically blocks the carrier in the cytoplasmic state (*c*-state) [30] and cannot pass through the membrane by passive diffusion. This means that the carrier must have been inserted into the membrane with the large matrix loops in the cytoplasm and with the N- and C-termini to the outside the cell. The insertion obeys the positive inside rule [23], even though the majority of bacterial proteins have the N- and C-terminal ends in the cytoplasm [31]. Furthermore, mitochondrial carriers are believed to form functional

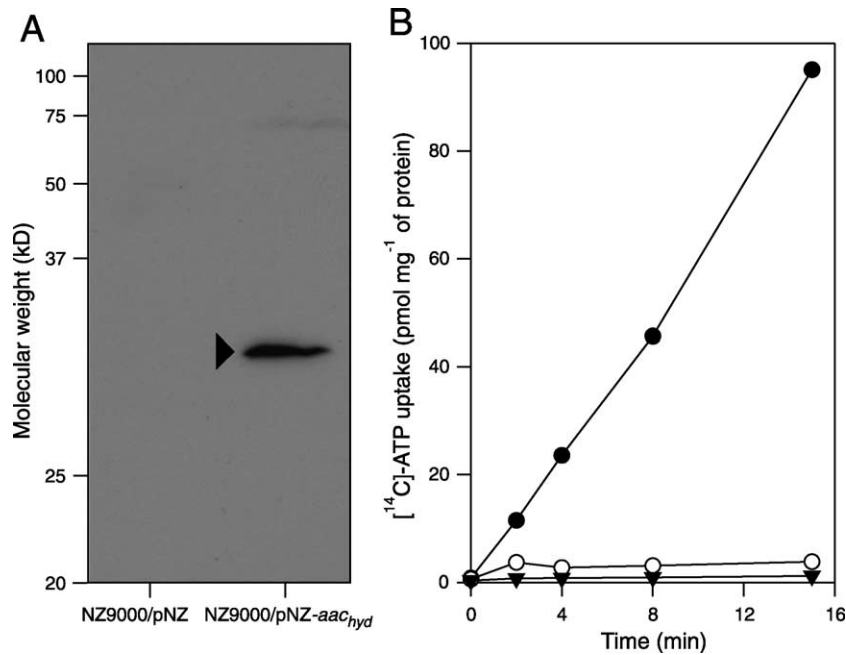


Fig. 6. Expression and functional characterisation of the *Neocallimastix* hydrogenosomal ADP/ATP carrier (AAC<sub>hyd</sub>). (A) Western blot of membranes isolated from nisin A-induced cells of the strain NZ9000 with vector control (NZ9000/pNZ) (lane 1) and of NZ9000 with the expression vector for the hydrogenosomal ADP/ATP carrier from *N. patriciarum* (NZ9000/pNZ-aac<sub>hyd</sub>) (lane 2). Membrane proteins were separated on SDS-PAA, transferred to PVDF membranes and probed with antibodies against the ADP/ATP carrier of *N. crassa* [28]. Approximately 20  $\mu$ g of total membrane protein was present in each lane. (B) [<sup>14</sup>C]-ATP uptake in whole cells of strain NZ9000/pNZ (open circles) and NZ9000/pNZ-aac<sub>hyd</sub> (closed circles) was started by diluting cells in buffer containing a final concentration of 1.54  $\mu$ M [8-<sup>14</sup>C]-adenosine 5'-triphosphate. At different time intervals, cells were separated from the assay buffer by filtration and the accumulated ATP was determined by scintillation counting. Carboxy-atractyloside (closed triangles) was added to a final concentration of 25  $\mu$ M.

homodimers, which must have occurred spontaneously in the lactococcal membranes. Mitochondrial carriers also require high levels of cardiolipin for function, which is abundantly present in lactococcal membranes up to 32% of total lipid [32].

We have also been able to overproduce the yeast mitochondrial ADP/ATP carrier 3 (AAC3) in a functional form in *L. lactis*. The initial transport rates of ATP into whole cells were directly dependent on the amount of nisin A used for the induction (Fig. 7A). In a CBB-stained SDS-PAA gel of isolated membrane fractions, a band was clearly visible at the appropriate molecular weight when cells were induced, while it was absent when nisin A was not added to the medium (Fig. 7B). The expressed AAC3 protein also reacted in Western blot analysis with antibodies raised against peptide 233–246 of the carrier (data not shown). These results illustrate that the *nisA* promoter is tight and can be regulated to express different amounts of membrane protein simply by varying the amount of inducer as was shown before for peptidase N [2].

In the final example, the expression of the yeast citrate carrier (CTP1) is shown. NZ9000 does not contain a functional citrate carrier nor does it carry out citrate metabolism. Expression trials clearly showed that the CTP1 carrier could also be expressed in the cytoplasmic membrane of *Lactococcus* (Fig. 8A). However, whole cells of the induced expression strain did not take up radiolabelled citrate, whether energised with glucose or not. This is likely to be

due to the absence of intracellular citrate or other suitable substrates that could be used for the exchange reaction. To test this hypothesis, membranes were isolated from the control and CTP1 expression strain, and fused with liposomes loaded with 5 mM citrate. Transport in membrane vesicles was initiated by diluting the fused membrane vesicles in buffer containing radioactive citrate in the presence of valinomycin and nigericin. Rapid uptake was observed in fused membrane vesicles isolated from the CTP1-expressing strain, while no uptake was observed in control membrane vesicles (Fig. 8B).

Two recent papers have shown that mitochondrial and hydrogenosomal AACs can also be functionally expressed in the cytoplasmic membrane of *E. coli* strains BL21 and C43 [29,33]. These two strains are routinely used for over-expression of mitochondrial carriers in inclusion bodies [27,34]. It is not clear what fraction of the carriers is targeted to the inclusion bodies rather than to the cytoplasmic membrane. Careful fractionation studies by urea and cholate or by carbonate extraction of peripheral proteins have shown that in *L. lactis* carriers are exclusively targeted to the cytoplasmic membrane, while inclusion bodies have never been observed (data not shown).

The rate of ATP transport by the hydrogenosomal AAC in *E. coli* cells was 840 pmol mg<sup>-1</sup> h<sup>-1</sup> at an external concentration of 50  $\mu$ M [29], while the rates in our study were around 400 pmol mg<sup>-1</sup> h<sup>-1</sup> at the external concentration of 1.54  $\mu$ M. However, it might not be correct to

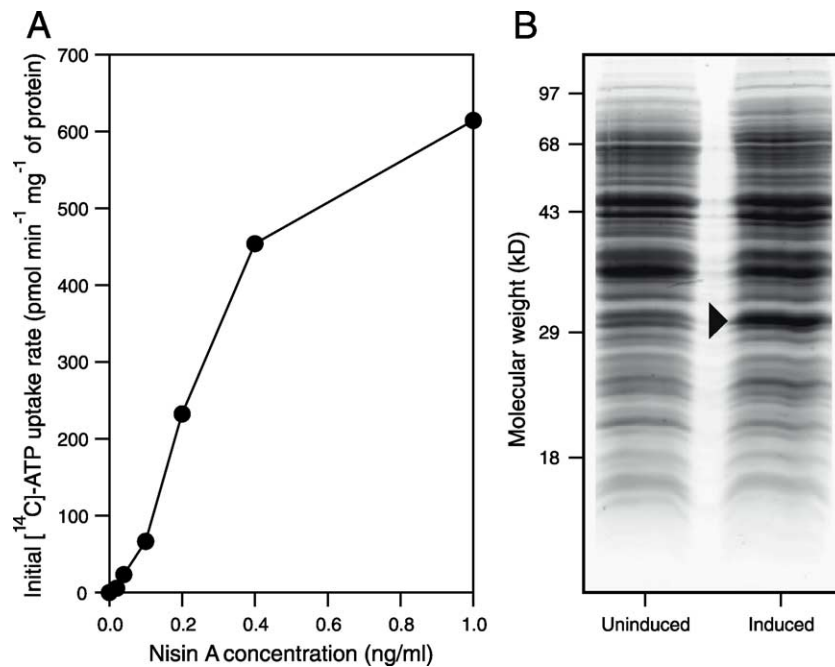


Fig. 7. Controlled expression of the yeast mitochondrial AAC3. (A) The initial uptake rates of [<sup>14</sup>C]-ATP in whole cells after induction with different amounts of nisin A. Cells were harvested by centrifugation, washed twice with PBS buffer and uptake was started by diluting cells in buffer containing a final concentration of 1.54  $\mu$ M [8-<sup>14</sup>C]-adenosine 5'-triphosphate. After 15 s cells were separated from the assay buffer by filtration and the accumulated ATP was determined by scintillation counting. (B) CBB-stained SDS-PAA gel of membranes isolated from uninduced and induced cells (1 ng/ml of nisin A) of strain NZ9000 harbouring the expression vector with the yeast *aac3* gene behind the *nisA* promoter (NZ9000/pNZ-*aac3*). Membranes were isolated by mechanical disruption and differential centrifugation. Approximately 30  $\mu$ g of total membrane protein was loaded in each lane.

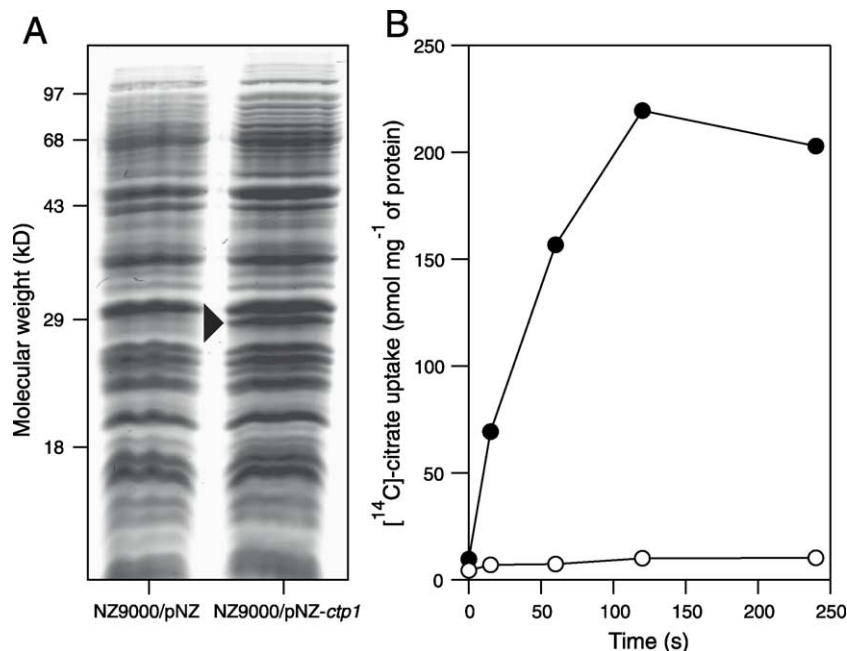


Fig. 8. Expression and functional characterisation of the yeast mitochondrial citrate carrier (CTP1). (A) CBB-stained SDS-PAA gel of membranes isolated from nisin A-induced cells of strain NZ9000 harbouring plasmid pNZ8048 (NZ9000/pNZ) (lane 1) or plasmid pNZ8048 with the yeast *ctp1* gene behind the *nisA* promoter (NZ9000/pNZ-*ctp1*) (lane 2). Approximately 40  $\mu$ g of total membrane protein was loaded in each lane. (B) Citrate counterflow in fused membrane vesicles isolated from strain NZ9000/pNZ (open circles) or NZ9000/pNZ-*ctp1* (closed circles). Prior to transport, membrane vesicles were fused with liposomes and loaded with 5 mM unlabelled citrate. Transport was started by diluting fused membrane vesicles in buffer containing 1  $\mu$ M valinomycin, 1  $\mu$ M nigericin and [1,5-<sup>14</sup>C]-citric acid at a final concentration of 1.2  $\mu$ M. At defined intervals, membranes were separated from the assay buffer by filtration and the accumulated citrate was determined by scintillation counting.

compare these rates directly, because (i) the external concentration of the substrate in our case is far below the apparent  $K_m$  and (ii) the internal ATP and ADP concentrations, which reflect the energy state of the cell and provide the driving force for transport, might have differed as well. Inhibitor studies in whole cells of *E. coli* are complicated by the presence of the outer membrane and do require the treatment of cells with lysozyme to remove the cell wall and possibly the outer membrane [33].

#### 4. Discussion

In this paper, we have shown that many integral membrane proteins can be successfully overproduced in a functional form in *L. lactis*. Examples that were presented included prokaryotic and eukaryotic membrane proteins from major membrane protein families, such as the major facilitators, the ABC transporters, the mechanosensitive channels, the mitochondrial carriers, the peptide transporters and the KDEL receptor.

The expressed proteins vary in size and number of predicted transmembrane spanning  $\alpha$ -helices, ranging from 2 to 12 helices. Furthermore, all proteins insert into the cytoplasmic membrane despite having different topologies with respect to the orientation of the N- and C-terminal ends. The proteins also assemble into functional complexes despite having different quaternary structures and/or domain organisations. In some cases, the expression of the membrane protein is toxic to the cell, but in many others, growth is completely unaffected as has been observed in other heterologous expression systems [24]. The expression levels vary from several micrograms to a few milligrams per liter, but in all cases, the membrane proteins were functional. So far, no inclusion bodies have been found and degradation of membrane proteins is minimal, despite the fact that protease inhibitors are not used during isolation and solubilisation of membranes.

The lactococcal genome is about half the size of that of *Escherichia*. This is perhaps an advantage as it may reduce the number of contaminants that need to be removed during purification. In a way, this may already constitute a purification step, but on the other hand, some chaperones and other proteins required for correct targeting and folding of more complex integral membrane proteins may be absent. For instance, the genome does not contain a gene coding for a disulfide isomerase. So far, we have not experienced limitations in the membrane protein assemblage machinery of *L. lactis*, because all overexpressed proteins were fully functional. Another possible concern is the AT-rich codon usage of the bacterium, which might prevent high expression levels. Strains could be constructed that overproduce tRNAs for rare codons to relieve this limitation. For soluble proteins that express poorly with the nisin expression system, the observation has been made that the levels can be improved significantly by fusing a DNA sequence,

corresponding to the 5' end of an highly expressed gene, to the gene of interest (I. Mierau, unpublished). Apparently, once the transcription apparatus has succeeded in rapidly transcribing the first  $\sim 10$  codons of a gene, the codon bias in the remaining part of the gene is less important. This method has been shown to improve the expression of many membrane proteins in *E. coli* [24]. In the case of the KDEL receptor, a synthetic gene was synthesized that replaced every codon of the human *erd2* gene for an AT-rich codon based on preferred codons of highly expressed lactococcal proteins. Also a mutant gene was constructed, in which only the first 15 codons were changed, but in both cases the expression levels of the KDEL receptor were 4-fold lower than observed with the original *erd2* gene (data not shown). The low levels of expression seen for the KDEL receptor are, therefore, probably not due to a limitation in the rate of translation, but most likely to the toxicity of overexpression which leads to rapid cell death. We are currently investigating a set of mutants that are resistant to the toxic effects of KDEL receptor expression in the hope to select strains that are capable of producing larger amounts of functional material.

The high success rate in the expression of functional membrane proteins, the possibility of studying the expressed transporters and receptors in intact cells, and the user-friendliness of *L. lactis* make further development of this expression system clearly worthwhile.

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